INOCULUMS DEVELOPMENT FOR THE PRODUCTION OF MONOCLONAL ANTIBODY AGAINST CONGENITAL ADRENAL HYPERPLASIA (CAH)

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ABSTRACT

By now, the importance of inoculums development was well appreciated in bioprocessing. Inoculums development receives as much care and attention as the growth and product cycles. The status or "health" of the inoculums has a great deal to do with the success of the production cycle. In this project, inoculums development for the production of monoclonal antibody against Congenital Adrenal Hyperplasia (CAH) was studied. The scopes focused were the age of inoculums (passage number) and inoculums density at different growth phases. A hybridoma cell line (Hybridoma 192) was cultivated in batch mode with varying inoculums cell densities and growth phases using Dulbecco's Modified Eagle Medium (DMEM). The maximum viable cell densities and the average specific MAb production rate (r_p) were found to dependent on the inoculums cell density and the growth phases. Inoculums with low cell density at $5x10^4$cells/ml and early log phase (t=28h) have the highest average specific MAb production rate but a low maximum viable cell density. Varying inoculums cell densites and growth phases did not affect the specific growth rate, maximum MAb titer and net production of MAb. Since the inoculums density in the range of $5x10^4$ cells/ml to $5x10^5$ cells/ml and growth phase do not affect the net production of MAb, it is wise to use high initial inoculums cell density at $5x10^5$ cells/ml because the cell growth easily and faster compare low inoculums density. For the inoculums age, the average specific MAb production rate decreased when the passage number increased from PN (Passage Number) =9 to PN=30.Subculture exceeding critical passage number 9 was thus not recommended.

ABSTRAK

Dalam projek ini, pembangunan inokulum untuk penghasilan monoklonal antibodi untuk menentang Congenital Adrenal Hyperplasia (CAH) telah dikaji. Skop yang difokuskan dalam pembangunan inokulum adalah umur inokulum, kepadatan sel inokulum dengan pelbagai fasa pertumbuhan hybridoma. Hybridoma dikulturkan dalam mode batch dengan pelbagai kepadatan sel inokulum dan fasa pertumbuhan dengan menggunakan Dulbecco's Modified Eagle Medium (DMEM). Daripada pemerhatian, maksimum kepadatan sel yang hidup dan purata spesifik kadar penghasilan MAb adalah bergantung kepada kepadatan sel inokulum dan fasa pertumbuhan. Inokulum yang mempunyai kepadatan sel yang rendah pada $5X10^4$ sel/ml dan awal fasa pertumbuhan mempunyai nilai yang tinggi dalam purata spesifik kadar penghasilan MAb tetapi nilai yang rendah dalam maksimum sel yang hidup. Kadar spesifik pertumbuhan sel, maksimum MAb titer dan jumlah bersih penghasilan MAb tidak bergantung kepada pelbagai kepadatan sel inokulum dan fasa pertumbuhan. Walaupun kepadatan sel inokulum dan fasa pertumbuhan tidak mempengaruhi jumlah bersih penghasilan MAb tetapi ia adalah senang dan cepat jika mengunakan kepadatan sel inokulum yang tinggi seperti $5x10^5$ sel/ml. Dalam umur inokulum, purata spesifik kadar penghasilan Mab menurun apabila nombor passage bertambah dari PN=9 ke PN=30. Kritikal nombor passage adalah penting , apabila sel menjangkau nombor passage ini, sel perlu dicairkan daripada sumber yang baru berbanding meneruskan sub-kultur di mana ia akan menpengaruhi penghasilan MAb.

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LIST OF ABBREVIATIONS

ABTS	2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
CAH	congenital adrenal hyperplasia
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOE	Design of Expert
EIA	Enzyme Immunoassay
FBS	Fetal Bovine Serum
IMEM	Iscove's modified Eagle's medium
MAb	Monoclonal antibidy
NCAH	non-classical congenital adrenal hyperplasia
PBS	phosphate-buffed saline
PES	polyethersulfone
PN	passage number
RIA	Radioimmunoassay
SDS	sodium dodecyl sulfate
17-OHP	17α-hydroxyprogesterone
21-OHD	21-hydroxylase

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Congenital Adrenal Hyperplasia (CAH) is a family inherited disorder seen in newborn which affecting the adrenal glands. The most common type, which occurs with a worldwide frequency of one per 15000 live births depending on the population, is associated with deficiency of 21-hydroxylase (21-OHD) , which is inherited in severe or mild forms. The severe form, called Classical Congenital Adrenal Hyperplasia, is usually detected in the newborn period or in early childhood. The milder form, called Non-classical Congenital Adrenal Hyperplasia (NCAH), may cause symptoms at anytime from infancy through adulthood. NCAH is a much more common disorder than Classical CAH. Fortunately, CAH can be managed with medication and, with adequate care; affected individuals go on to live normal lives.

CAH is an autosomal recessive genetic disorder. It affects males and females in equal numbers. These particular groups of genes contain instructions the adrenal glands (located on top of the kidneys) need in order to produce an enzyme called 21-hydroxylase. Without this enzyme, the adrenal glands are unable to produce cortisol, a hormone necessary for life.

Cortisol is a steroid produced by the adrenal glands that our bodies need to deal with physical and emotional stress, and maintain adequate energy supply and blood sugar levels. The adrenal glands are controlled by the pituitary gland. The pituitary gland is a small pea-sized gland at the base of the brain. When the pituitary gland senses that there is not enough cortisol present in the bloodstream, it releases a hormone called ACTH (adrenocorticotropic hormone). ACTH stimulates the adrenals to produce more cortisol. However, those with CAH have insufficient amounts of the enzyme 21-hydroxylase, needed to convert a precursor molecule called 17-hydroxyprogesteron (17-OHP) into cortisol. As a result, the pituitary gland continues to sense the need for cortisol and pump out more ACTH. This leads to an overabundance of 17-OHP, which is converted in the adrenals into excess androgens (masculinizing steroid hormones). Lack of adequate cortisol also prevents the body from properly metabolizing sugar and responding to stress. The lack of this stress response can lead to an adrenal crisis.

The measurement of 17α-hydroxyprogesterone (17OHP) in serum or plasma is clinically useful for the diagnosis and management of congenital adrenal hyperplasia (CAH). The conventional method used for measuring 17OHP is conventionally based on competitive-binding radioimmunoassay or RIA (4, 5), using polyclonal antibody and usually require solvent extraction of samples. However, in the past decade clinics are learning more towards into using Enzyme immunoassays (EIA) for easier mass screening of steroids in patients' serum or plasma. EIA possesses several advantages over RIA. These include the avoidance of problems associated with the use of radioisotopes, the shorter incubation period and less work involved in the overall procedure. Additionally, immunoassay are now increasingly based on monoclonal antibodies (MAbs) instead of antisera (polyclonal antibodies). This is due to the fact that MAbs possess uniform characteristics, are well defined proteins and are readily available in unlimited supplies (H. Chong et al., 2009).

1.2 Problem statement

Hybridoma technology which is established by Kohler and Milstein had been used and one of the cell line (Hybridoma 192) tested successfully to produce monoclonal antibody against Congenital Adrenal Hyperplasia (CAH) which is anti 17-hydroxyprogesteron (17-OHP).The demand for the monoclonal antibody is high but the production or yield of monoclonal antibody by hybridoma is low. The scales-up productions of monoclonal antibody are difficult and cause the cost of the process becomes very expensive. Besides that, during the cell culture, hybridoma cell have a high chance to contaminated and make the process more difficult. The main problem in the considerations is how to optimize the production of monoclonal antibody and second and how to ensure that the entire hybridoma cell is active and healthy. To solve this problem, inoculums development plays important roles in the initial stage to scale up the monoclonal antibody production.

1.3 Objectives of Study

In the course of completing this project, there are few objectives to be fulfilled. There are

1. To check the effect of inoculums age to the production of monoclonal antibody and cell growth. If it does affect, the maximum inoculums age that can be used in monoclonal antibody production will be determined.
2. To check the effect of inoculums density and growth phase to the production of monoclonal antibody and cell growth. The suitable density that can be used in monoclonal antibody production will be determined.

1.4 Scope of Study

Based on the objective, the main scope of this project is inoculums development in production of monoclonal antibody by hybridoma cell. The scope

including inoculums age, inoculums density and growth phase. For inoculums age, area that was study based on number of subcultures or passage number (30 times) of hybridoma cells on the production of monoclonal antibody and cell growth. For inoculums density and growth phases, the effect of different density($5x10^4$-$5x10^5$ cells/ml) together with different growth phases (early log phase, mid log phase and late log phase) of hybridoma cell on the production of monoclonal antibody and cell growth.

1.5 Rational and Significance

Inoculums development is crucial to the initial stage of scale up the monoclonal antibody production. Through inoculums development, we can increase the quality of inoculums and this ensures the successful of the run during the scale-up production. From this, we can minimize the lost of money by optimize the yield of monoclonal antibody. The production time which is very valuable to the industry can become shorter when the yields increase.

CHAPTER 2

LITERATURE REVIEW

Animal cells are increasingly grown in bioreactors for the production of vaccines and proteins. In such systems the growth and metabolic activity of cells depends on many parameters: the chemical composition of the medium, the initial physiological state of the cells, the physico-chemical conditions during the culture (Griffiths, 1986).In recent years substantial quantitative data have been obtained on the influence of the chemical composition of the medium on the kinetics of growth and metabolism of several cell lines (Dalili and Ollis1989; and Geaugey et al., 1989).The influence of operational parameters such as pH, dissolved oxygen, osmotic pressure and redox potential have also been reported (Miller et al., 1988 and 1987).Little has been published , however on the inoculums development.

The handling of a microbial culture, from the time it is transferred from its preserved state until it is inoculated into the final location where the microbial activity of interest is expressed, is referred to as inoculums development. The primary purpose of inoculums development is to provide microbial mass, of predictable phenotype, at a specific time, and at a reasonable cost for the productive stage of a microbial activity. Until now, inoculums development has been more art than science. There remains a need especially at the shake flask or spore-generating stages of the process, for time and ‘it looks good’ criteria to be replaced with biochemical, physiological, or morphological markers as both descriptions of an optimum and indicators for optimum timing of inoculums transfer.

It is essential that the culture used to inoculate a fermentation satisfies the following criteria:

1. It must be in healthy, active state thus minimizing the length of the lag phase in the subsequent fermentation.
2. It must be available in sufficiently large volumes to provide an inoculums of optimum size.
3. It must be in a suitable morphological form.
4. It must be free of contamination.
5. It must retain its product-forming capabilities.

The process adopted to produce an inoculums meeting these criteria is called inoculums development. Hockenhull is credited with the quotation "once fermentation has been started it can be made worse but not better" (Calam, 1976). Where this is an over-statement it does illustrate the importance of inoculums development. Much of the variation observed in small-scale laboratory fermentations is due to poor inocula being used, thus, it is essential to appreciate that the establishment of an inoculums development programme is equally important regardless of the scale of the fermentation. Such a programme not only aids consistency on a small scale but is invaluable in scaling up the fermentation and forms an essential part in progressing a new process (Freshney, 2005). Criteria above that apply in microbe fermentation also apply in cell culture.They are equally important either to cell culture or microbe fermentation.

Figure 1.1 shown the inoculums growth of hybridoma in T flasks. A T flask is inoculated with $2X10^5$ cells/ml harvested at the end of 48 hr of propagation. An initial fast growth period up to $10X10^5$ cells/ml is observed during the first 50 hr. Cell density reaches a maximum at about 60 hr, and then slowly decreases. The first 24 hr , the cells undergo lag phase after that the log phase begin until the cell density reach the maximum at 60 hr , and the cell undergo death phase. This is the growth profile of a typical hybridoma cells (Martial et.al, 1990).

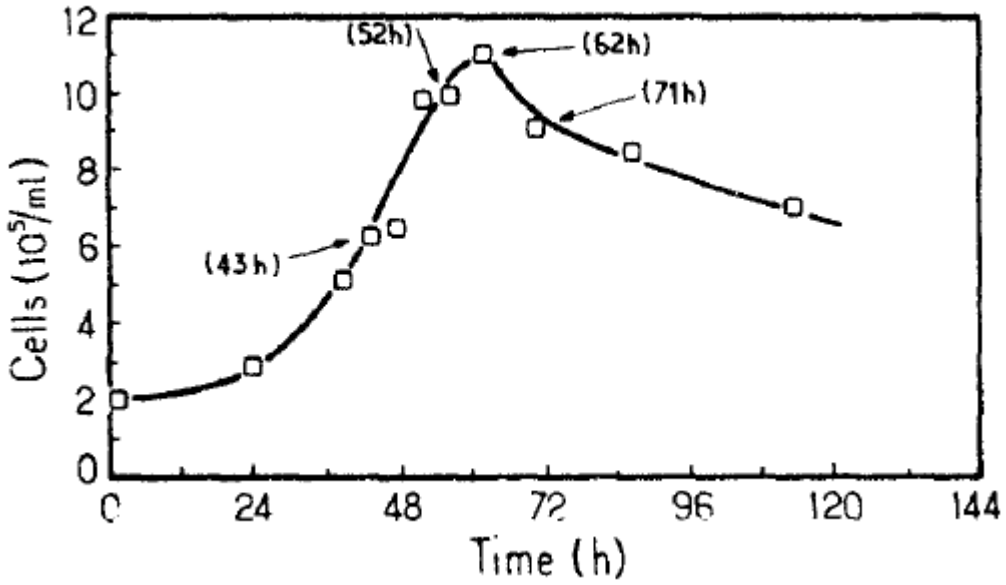

Figure 2.1: Evolution with time of the viable cell density during the hybridoma T flask culture (Martial et.al, 1990).

Several research have been done in inoculums development in hybridoma culture, one of it is to check the influence of inoculums age on hybridoma culture. Where the inoculums ages that used is 43hr (T1), 52hr (T2), 62hr (T3), and 71hr (T4). They investigated in four different spinner flasks inoculated at the same initial density of $2x10^5$ cell/ml but with the cells having been propagated in a T flask for the above culture durations. In Figure 1.2, they found that T1 and T2 (43 hr and 52 hr respectively) present two usual growth and death phases of a batch culture, without any appreciable initial lag phase. On the contrast, spinners T3 and T4 (62 hr and 71 hr respectively) exhibit an initial lag phase lasting as long as 45 hr. The viability of the cells, which is initially around 90% for the three first spinners, remain high during the whole growth phase and then declines during the death phases. For the oldest inoculums, the viability remains around 70% during both the lag and growth phases (Martial et.al, 1990).These shows that inoculums age that used in stationary phase and death phase will cause the decrease of maximum viable cell density, maximum specific growth rate and cell viability (%).

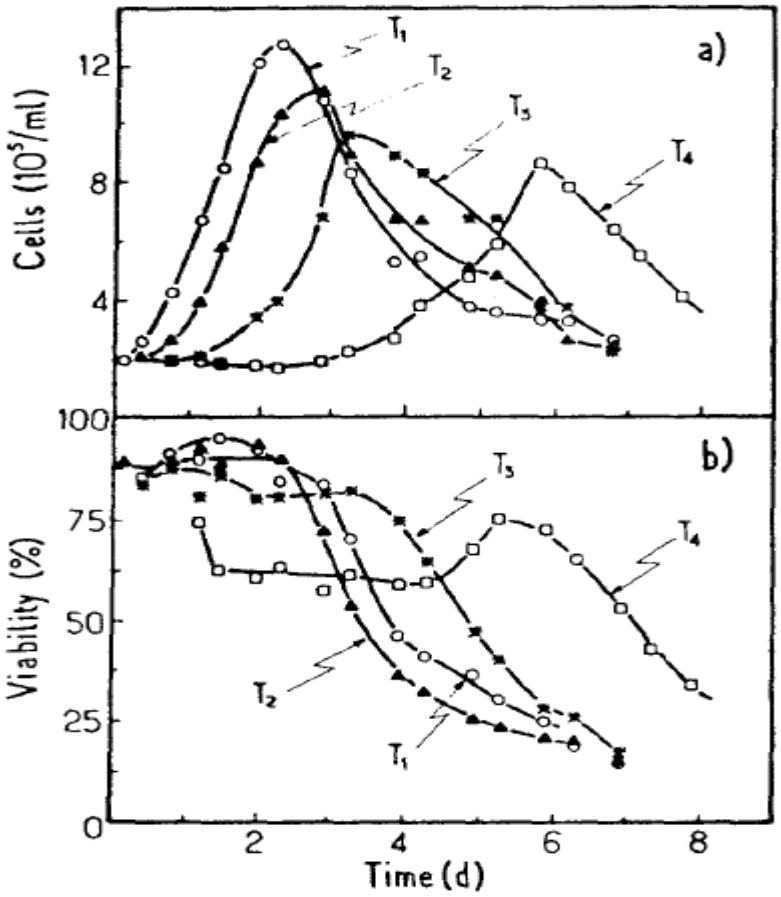

Figure 2.2: Evolution with time of the viable cell density (a) and the viability (b) for hybridoma cultures in spinner flasks with different inoculums ages (T1:43 hr; T2:52 hr; T3:62 hr; T4:71 hr) (Martial et.al, 1990).

Figure 1.3 shows the different inoculums age on production of MAb. Although the figure shows that when the inoculums age increase, the production of MAb decrease in the early stage and no antibodies are produced during lag phase especially in T4 but the final antibody levels are essentially the same. Since less cells are produced starting from an old inoculums, they conclude that these cells have a higher specific production rate of antibodies (Martial et.al, 1990).

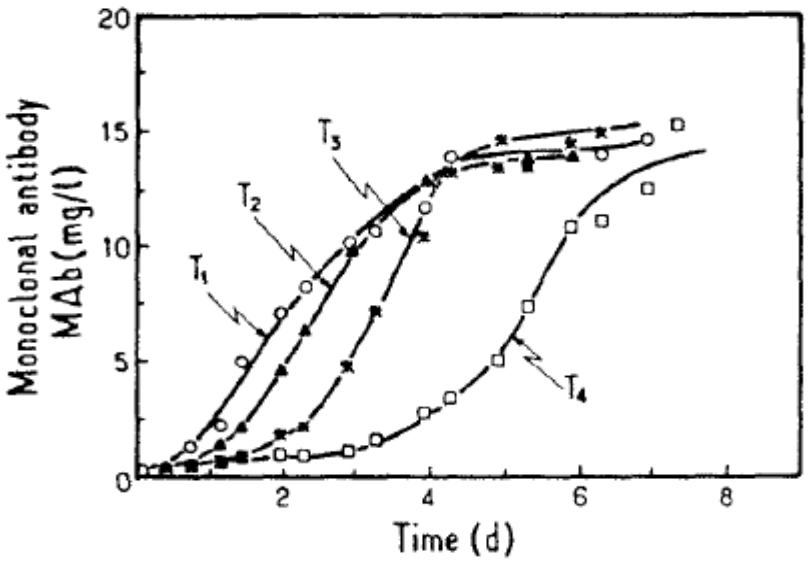

Figure 2.3: Kinetics of monoclonal antibodies production for hybridoma cultures in spinner flasks with different inoculums ages (T1:43 hr; T2:52 hr; T3:62 hr; T4:71 hr) (Martial et.al, 1990).

For the effect of initial density on hybridoma growth and monoclonoal antibody production which done by Ozturk and Palson (1990), the range of density used is10^2 to 10^5 cells/ml. They cultured the murine hybridoma, 167.4G5.3 in 75 cm^2 plastic T-flasks with different inoculums density and serum concentration (1.25, 2.5, 5, and 10% v/v). Figure 1.4, shows the maximum viable cell concentrations and maximum IgG antibody concentration for hybridoma culture in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with different serum concentration at different initial cell densities. In this figure, it shows that increase the initial cell density will increase the maximum viable cells and also maximum antibody concentration.

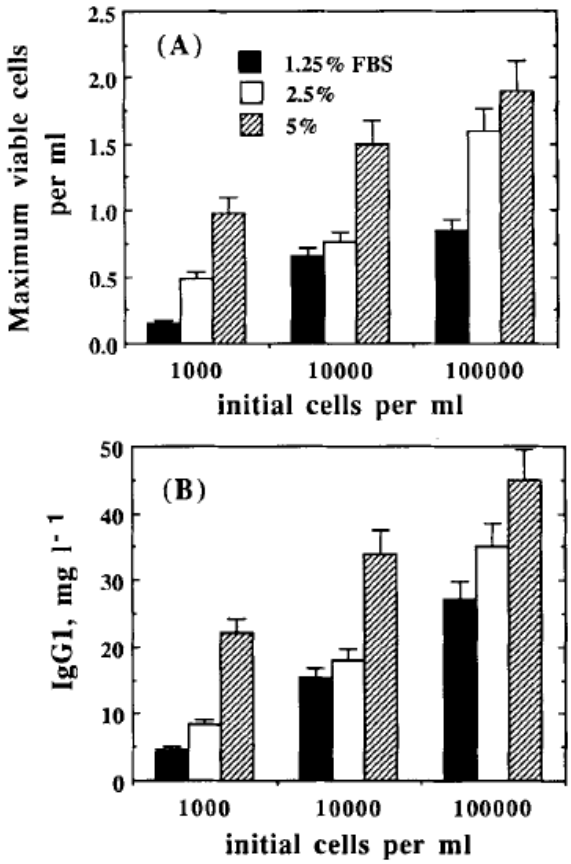

Figure 2.4: (A) Maximum cell and (B) maximum IgG concentrations for hybridoma cell line 167.4G5.3 in cultures with different serum levels and with different initial cell densities (Ozturk and Palsson, 1990).

Monoclonal antibody concentration increased gradually in all the batches following the viable cell count. More antibodies were produced for the cultures with higher serum and higher initial cell concentrations. Antibody production continued into the decline phase of growth. From the Figure 1.4 (B), a ten-fold increase in inoculums size resulted in only a two-fold increase in final antibody concentration. From this research, Ozturk and Palson concluded that the maximum viable cell antibody concentrations are determined by inoculums sized. The changes in antibody concentration were due to changes in cell concentration as the specific antibody production rate was not altered (Ozturk and Palsson, 1990).

CHAPTER 3

METHODOLOGY

3.1 Basal Medium

Around 70 to 80% of 1 L ultrapure water was poured into a 2L beaker. 13.4 grams of Dulbecco's Modified Eagle Medium (D5648, Sigma-Aldrich) was dissolved slowly with gentle stirring by magnetic stirrer. The residue in the weighing boat was rinsed with little ultrapure water. The solution was stirred for at least 30 minutes. 3.7 g of sodium bicarbonate (S5761, Sigma-Aldrich) was then added and allowed to mix for 10 minutes. 1 g of pluronic F-68 (P1300, Sigma-Aldrich) was also added and mixed for further 10 minutes. The volume of the solution was brought to 1 liter by adding the remainder ultrapure water. The pH was adjusted to 7.2 by using 0.1N hydrochloric acid. The solution was mixed for another 30 minutes. 17.28µM sodium selenite, 4.47µM zinc sulfate and 25.6µM ferric citrate were then added to the solution. The solution was mixed and sterile filtered with 0.22µm polyethersulfone (PES) membrane filter (F-99505, TPP) into the 1L of Scott bottle by using vacuum pump. The filtered medium (basal medium) was kept in the chiller (4˚C) for a maximum of 2 months.

3.2 Complete Medium

To prepare 400 ml complete medium for cell culture, 1.6 ml of fetal bovine serum (10091-148, GIBCO), 4 mM L-glutamine (non-animal source, G8540, Sigma-Aldrich), and 4 ml of antibiotic-antimycotic (15240-062, GIBCO; 1×) were added to a sterile 500ml Scott bottle. Basal medium was then added until the volume reach 400 ml. The complete medium was kept in the chiller (4˚C) for a maximum of 2 weeks.

3.3 Thawed Cell

Cells were thawed by warming a vial of cells in the water bath (35˚C) for a maximum of 1 min or until it is partially melt, immediately after removing from the -80°C freezer. Thawed cells were quickly diluted into a 25 ml warm complete medium. The cells were centrifuged (Eppendorf, Germany) at 120*g* for 5 minutes at 4˚C. After centrifugation, the supernatant was discarded and the cells were resuspended with 25 ml fresh medium. The cells were then transfered to a T-75 tissue culture flask and incubated in a humidified CO_2 incubator (Shel Lab, United State) at 5% CO_2 and 37°C.

3.4 Cell Count

Before cell count, the tissue culture flask was tapped to dislocate and mix all the cells evenly. Hemocyctometer slide and the coverslip were cleaned with the 70% ethanol. Kimwipe paper was used to wipe the hemocyctometer slide and cover slip. The cell sample was mixed and 20µl sample was transferred to a 0.5 ml centrifuge tube. Twenty µl trypan blue dye (for a dilution factor of 2×) was transferred to the tube and mix with the sample. Ten µl of this mixture was transferred to the edge of the hemocytometer chamber. The cell suspension was drawn under the coverslip by capillary action. This was repeated for the other chamber. The hemocytometer was